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From: Hunt, Jennifer
Sent: Sunday, November 04, 2001 12:29 PM
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Subject: References for 09/218,481

Please send me the following references ASAP:

JOURNAL OF CEREBRAL BLOOD FLOW AND METABOLISM, (1998 Aug) 18 (8) 887-95

Adv. Exp. Med. Biol. (1998), 454(Oxygen Transport to Tissue XX), 311-317

BRAIN RESEARCH. MOLECULAR BRAIN RESEARCH, (1998 Sep 18) 60 (1) 89-97

JOURNAL OF APPLIED PHYSIOLOGY, (1998 Jul) 85 (1) 53-7

STROKE, (1997 Oct) 28 (10) 2039-44

NEUROSURGERY, (JUN 1997) Vol. 40, No. 6, pp. 1269-1277

NEUROSURGERY, (1997 May) 40 (5) 1016-26

JOURNAL OF CLINICAL INVESTIGATION, (1996 Sep 15) 98 (6) 1400-8

JOURNAL OF NEUROSURGERY, (1996 Dec) 85 (6) 1095-101

Proc Annu Meet Am Assoc Cancer Res, (1996). Vol. 37, pp. A399

Oncology Reports, (1995) 2/6 (1147-1149)

NEUROSURGERY, (1994 Sep) 35 (3) 439-48

YALE JOURNAL OF BIOLOGY AND MEDICINE, (1993 Jul-Aug) 66 (4) 277-314

MOLECULAR BIOLOGY OF THE CELL, (1993 Jan) 4 (1) 121-33

Endothelial Cell Dysfunct. (1992), 477-503

J CELL BIOL. (1990) 111 (5 PART 2), 227A.

Thanks,

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Rat brain VEGF expression in alveolar hypoxia: possible role in high-altitude cerebral edema

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Xu, Fengping, and John W. Severinghaus. Rat brain VEGF expression in alveolar hypoxia: possible role in high-altitude cerebral edema. *J. Appl. Physiol.* 85(1): 53–57, 1998.—The mechanism by which hypoxia causes high-altitude cerebral edema (HACE) is unknown. Tissue hypoxia triggers angiogenesis, initially by expressing vascular endothelial growth factor (VEGF), which has been shown to increase extracerebral capillary permeability. This study investigated brain VEGF expression in 32 rats exposed to progressively severe normobaric hypoxia (9–6% O_2) for 0 (control), 3, 6, or 12 h or 1, 2, 3, or 6 days. O_2 concentration was adjusted intermittently to the limit of tolerance by activity and intake, but no attempt was made to detect HACE. Northern blot analysis demonstrated that two molecular bands of transcribed VEGF mRNA (~3.9 and 4.7 kb) were upregulated in cortex and cerebellum after as little as 3 h of hypoxia, with a threefold increase peaking at 12–24 h. Western blot revealed that VEGF protein was increased after 12 h of hypoxia, reaching a maximum in ~2 days. The expression of *flt-1* mRNA was enhanced after 3 days of hypoxia. We conclude that VEGF production in hypoxia is consistent with the hypothesis that angiogenesis may be involved in HACE.

angiogenesis; cytokines; brain capillary leak; acute mountain sickness

HIGH-ALTITUDE CEREBRAL EDEMA (HACE) is one form of severe acute mountain sickness. The pathophysiological link between hypoxia and HACE is poorly understood. Pathological findings include retinal and presumably other cerebral petechial hemorrhages, cerebral thrombosis, and brain edema (21). The possibility that hypoxia might initiate angiogenesis in brain and underlie HACE was supported by the finding of increased capillary density in hypoxic rat brain (9, 14, 15) and by the observation that dexamethasone, widely used to prevent and treat HACE, is an effective blocker of angiogenesis (21).

Tissue hypoxia is thought to upregulate a series of local factors that contribute to angiogenesis, the growth of new capillary vessels. A complex cascade of cellular responses, triggered by local hypoxia, increased lactate, and/or low redox state, results in capillary basement membrane dissolution and rupture, as well as plasma and red blood cell extravasation. Endothelial cell budding and growth toward the hypoxic region normally follow. In recent years, many putative angiogenic factors have been identified, including vascular endothelial growth factor (VEGF), epidermal growth factor, transforming growth factors-α and -β, tumor angiogenesis factor, angiogenin, tumor necrosis factor-α, acidic and basic fibroblast growth factors, platelet-derived endothelial cell growth factor, and interleukin-8. Among these, VEGF is thought to be the most potent and specific in the basement membrane destruction and leakage. VEGF has been described as a specific in vitro endothelial cell mitogen and as an angiogenic inducer in several in vivo models (1). It is also known as a vascular permeability factor by virtue of its permeability-enhancing effects that, on a molar basis, enhanced the permeability of normal venules and small veins with a potency some 50,000 times that of histamine (20). VEGF has been shown to be upregulated by hypoxia both in vitro and in vivo (17, 24). It has not previously been sought during systemic hypoxia in brain where the blood-brain barrier might exclude such protein cytokines.

Angiogenesis in the brain normally occurs only during growth (19). Endothelial cell proliferation is low in the adult brain. Angiogenesis can occur in brain under pathological conditions such as infarction and tumor growth. VEGF has been detected in brain tumor tissue and was reported to be expressed in rat cerebellum and mouse choroid plexus (3, 16) and after surgical trauma (tumor removal) (21). Cerebral venous thrombosis, a complication of HACE, is consistent with the ability of VEGF to increase von Willebrand factor release (4) and thromboplastin activity (7).

We report here the expression of both VEGF mRNA and VEGF protein in rat brain as a function of time of inhalational hypoxic exposure, and we suggest that this may contribute to HACE.

MATERIALS AND METHODS

Animal experiments. All studies had prior approval of the committee on Animal Research, University of California, San Francisco. Adult Sprague-Dawley rats (Hilltop Strain, Banpin & Kingman) of either sex (weight 280–300 g) were housed in an aquarium with a plastic cover, kept in normal circadian rhythms (dark at night), and were supplied with food and water. A continuous fresh gas flow was supplied to the chamber, keeping the CO_2 concentration below 1%. Ambient O_2 concentration was reduced to ~9% initially, and it was reduced progressively over the next hours, in response to activity, to as low as 6%. Generally, the O_2 concentration was lowered ~1% every 3 h during the first day until the animals showed little activity and a decreased intake of food and water. Eight rats were housed in the same chamber for each group to ensure equal hypoxic exposures for all animals. The rats were harvested one at a time after eight hypoxic exposures of 0 (control), 3, 6, or 12 h or 1, 2, 3, or 6 days. The procedure was repeated for a total of four runs, each with eight rats. General appearance, activity, response to stimuli, and intake and excretion were recorded daily. O_2 and CO_2 concentrations were continuously monitored over the 6-day period with use of a PDP 11/44 computer and Perkin Elmer 1100 mass spectrometer. The rats were anesthetized with halothane and then decapitated. Brains were quickly re-

moved, frozen with liquid nitrogen, and stored at −80°C. Brain edema was not quantified.

Biochemical and biological reagents. Recombinant human $VEGF_{165}$ ($rhVEGF_{165}$) (R & D Systems, Minneapolis, MN) was diluted with PBS/BSA (0.3%) to 1 μg/5 ml, aliquoted, and stored at −80°C. VEGF cDNA (393 bp cloned into pGEM3 plasmid) was a gift from Dr. Larry Brown (Beth Israel Hospital, Boston, MA). *Flt-1* cDNA (458 bp in pGEM3Z) was a gift from Dr. Rubin M. Tuder (Department of Pathology, University of Colorado Health Sciences Center). Mouse β-actin (Ambion, Austin, TX) is a linearized pTRPLEscript plasmid containing a 250-bp mouse β-actin gene fragment. 28S ribosome (Ambion) is a linearized pTRPLEscript plasmid containing a 115-bp cDNA fragment of the human 28S rRNA gene. Polyclonal rabbit antibody against rhVEGF was purchased from Santa Cruz Biotechnology. Anti-rabbit IgG conjugated with horseradish peroxidase was obtained from Vector Laboratories (Burlingame, CA). The 0.24- to 9.5-kb RNA ladder used in Northern blot analysis was obtained from GIBCO-BRL Life Technologies. The rainbow-colored protein molecular-weight marker used in Western blot analysis was purchased from Amersham Life Science.

Northern blot hybridization. Total RNA was isolated from 100-mg pieces from cerebral cortex of rat brain by using a single-step method. Tissue was homogenized in 1 ml of RNA STAT-60 (TEL-TEST"B", Friendswood, TX) by using polytron homogenizer. The total RNA was extracted with chloroform and precipitated with isopropanol followed by a wash with 70% ethanol. The RNA pellet was dissolved in Tris-EDTA buffer (pH 7.5), and the optical density was determined by Shimadzu Recording Spectrophotometer UV-1601. Ten micrograms of total RNA were denatured at 65°C in formamide- and bromide-containing loading buffer and were subsequently electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde in 1× MOPS-EDTA-NaOAc buffer. RNA was transferred to nylon membranes (Hybond N, Amersham International PLC, Aylesbury, Bucks, UK) in 20× NaCl-NaH_2PO_4-EDTA buffer. Blots were cross-linked by ultraviolet irradiation (UV Statalinker, Stratagene), prehybridized in a seal-a-meal bag at 50°C for 1 h in prehybridization solution (1 M NaCl, 1% SDS), and then hybridized at 65°C overnight in hybridization solution (1 M NaCl, 1% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA). The cDNA probes used for hybridization were labeled with [^{32}P]dCTP (New England Nuclear, Boston, MA) to a specific activity of $1–2\times10^9$ counts · min^{-1} · μg DNA^{-1} by using the random dexamer labeling method (Redprime DNA Labeling System, Amersham). Denatured labeled DNA probe was added to the hybridization solution to a final concentration of 1×10^6 counts · min^{-1} · ml^{-1}. After overnight hybridization, the blots were washed twice in 2× NaCl-NaH_2PO_4-EDTA buffer with 0.1% SDS at room temperature for 5 min each, and then at 65°C for 5–30 min, and were exposed overnight to X-ray film with intensifying screens at −80°C. Blots probed for VEGF mRNA were stripped by boiling in 0.1% SDS for 5 min, and the blot was left in the solution until the solution returned to room temperature. All the blots were reprobed for β-actin and 28S antisense RNA probe in a similar manner to permit loading and blotting differences between lanes to be compensated. β-Actin and 28S RNA probes were prepared by T7 RNA polymerase and labeled with [^{32}P]UTP (>2,000 Ci/mmol; New England Nuclear, Boston, MA) with the use of MAXIscript in vitro transcription kits (Ambion). The intensity of the signals was quantified by a scanning densitometer.

Western blot analysis. Samples (100 mg) of rat brain cortex from the same brains used for Northern blot analysis were homogenized thoroughly in 1 ml of lysis buffer (0.01 M Tris · HCl, pH 7.6, 0.1 M NaCl, 0.1 mM dithiothreitol, 0.001 M EDTA, 0.1% NaN_3, 1 μg/μl leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/μl aprotinin, 1% NP-40). The extracts were centrifuged in a microfuge at 12,000 *g* for 5 min to remove particles. Total protein was determined by using bicinchoninic acid protein assay reagent (Pierce, Rochford, IL). Forty micrograms of total protein dissolved in sampler buffer containing 2-mercaptoethanol were loaded into each individual lane. Twenty micrograms of 1 μg/ml rhVEGF were used as a positive control. The proteins were then separated by 10% SDS-PAGE at 4°C at 15 mA for 30 min followed by 20 mA for 2–3 h until the blue dye reached the bottom of the gel. The proteins were then transferred onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham) with constant current of 200 mA at 4°C for at least 4 h. After transfer, the nitrocellulose blot was blocked overnight with 10% solution of dry milk to prevent nonspecific staining. It was then incubated for 1 h at room temperature with polyclonal antibody against rhVEGF, at a 1:500 dilution, in 10% dry milk in Tris-buffered saline/0.1% Tween, with gentle agitation. Subsequently, the filter was rinsed several times and incubated for 1 h at room temperature with anti-rabbit IgG horseradish peroxidase conjugate at a dilution of 1:20,000 in 1% solution of dry milk. Immunoreactive proteins were detected with use of the ECL Western Blotting Detection System (Amersham). The membrane was exposed to Hyper film ECL (Amersham) at room temperature for 5 min to 2 h.

Statistics. Results are expressed as a ratio of relative intensity of VEGF to β-actin at the corresponding time point. Mean values for total VEGF/β-actin, upper bands of VEGF/β-actin, as well as lower bands of VEGF/β-actin were compared by unpaired Student's *t*-test statistical analysis. Results were considered as statistically significant at $P < 0.05$.

RESULTS

Expression of VEGF mRNA in response to hypoxia in vivo. Figure 1 shows autoradiographs of Northern blots of total RNA from rat brain cortex for one run, with samples after each of the eight test periods. The *top* panel shows the hybridization signal for VEGF, the *middle* panel shows the signal for mouse β-actin, and the *bottom* panel shows the signal for the 28S ribosome. RNA from rat brain exhibited two hybridization signal bands for VEGF at ~3.9 and 4.7 kb. Hybridization patterns with RNA for β-actin were used as an index of the amount of total RNA applied to each lane. 28S ribosome was also used to correct for loading variation of total RNA on each lane. To normalize the data between individual blots, the relative intensities for the hybridization signal (intensity of VEGF mRNA signal divided by intensity of respective β-actin or 28S) are presented in Fig. 2. There are significant ($P < 0.05$) differences in the relative intensities of both 3.9 and 4.7 kb, as well as the total VEGF mRNA signal between the normal and hypoxic rat brains. No differences were observed between the relative intensities when β-actin was used as reference and those when 28S was used as reference.

After the rats were exposed to hypoxia for as little as 3 h, their VEGF mRNA levels were remarkably increased, reaching a maximum at ~12 h. Brain VEGF mRNA increased about threefold within the first 24 h. Scanning densitometric analysis showed that the maxi-

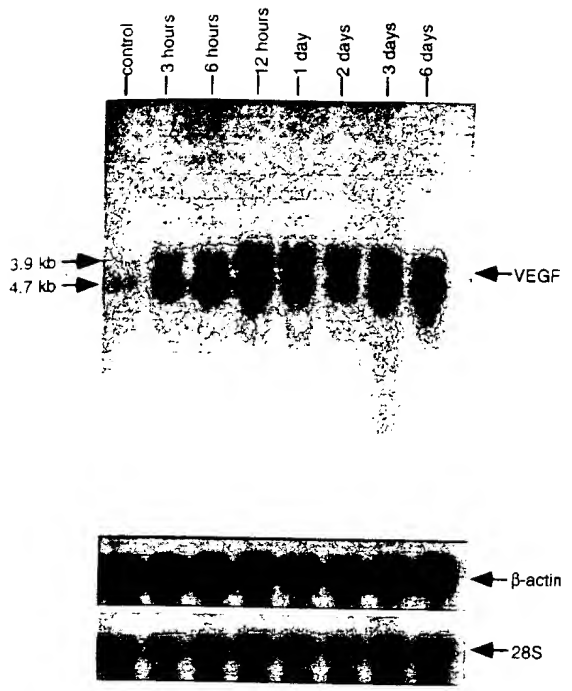

Fig. 1. Expression and response to hypoxia of vascular endothelial growth factor (VEGF) mRNA in rat brain. Total RNA was extracted from normal rats (control) or rats exposed to 9–6% O_2 for 3 h–6 days. Total RNA (10 μg) was loaded in each lane. Northern blots of total RNA sequentially hybridized with VEGF (*top*), β-actin (*middle*), and 28S (*bottom*) probes are shown.

mal effect occurred in 12–24 h. Enhanced VEGF mRNA persisted for at least 6 days.

Induction of VEGF protein production by hypoxia. We performed SDS-PAGE under reducing conditions and Western blot analysis on homogenized whole brain to detect VEGF protein as expected with VEGF mRNA expression. As shown in Fig. 3, one protein band at 23 kDa was detected with use of a polyclonal antibody to human VEGF. VEGF in rat brain was not increased until 12 h of hypoxia, although the VEGF mRNA increased as early as 3 h in hypoxia. A maximum induction of VEGF protein was reached after 2 and 3 days of hypoxia.

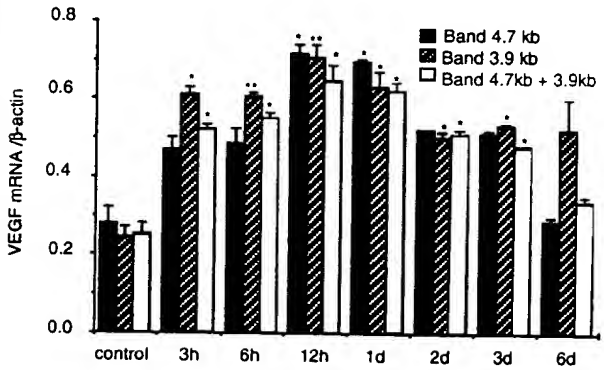

Fig. 2. Effects of hypoxia on VEGF mRNA expression in rat brain. Means ± SE are shown of VEGF mRNA optical density corrected for β-actin in rat brains removed during phases shown on horizontal axis. d, Day. Significantly different from control: $^{*}P < 0.05$, $^{**}P < 0.01$.

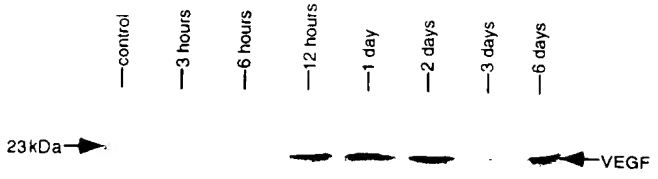

Fig. 3. Western blot analysis of brain tissue extract of normal rat (control) and rat exposed to hypoxia for 3 h–6 days. Total protein (40 μg) resolved under reducing condition (100 mM of 2-mercaptoethanol) was loaded in each lane and separated by 10% SDS-PAGE. Nitrocellulose blot was immunostained with polyclonal antibody against recombinant human VEGF. Band at 23 kDa is consistent in size to monomer of $VEGF_{165}$.

Upregulation of flt-1 mRNA during hypoxia. Northern blot analysis was performed for the mRNA expression of *flt-1*, one of the two VEGF receptors, in normal and hypoxic rat brain tissue. Figure 4 showed that *flt-1* mRNA was induced in rat brain after 3 days of hypoxia but then fell despite constant severe hypoxia.

DISCUSSION

Hypoxic exposure of awake rats induced a significant increase in brain VEGF mRNA expression as well as VEGF protein production. Interestingly, VEGF was upregulated primarily during the first few days of continued hypoxia, similar to the time course encountered in the development of symptoms and signs of HACE in humans ascending too rapidly to high altitude. The expression of VEGF mRNA was increased within 3 h of hypoxia, reached a peak at 12–24 h, and then declined. The production of VEGF protein peaked

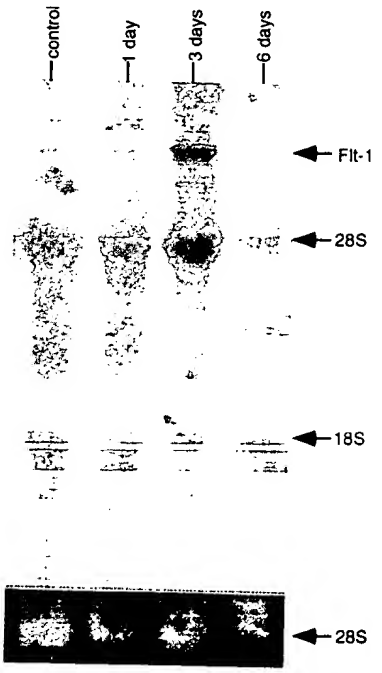

Fig. 4. Expression of *flt-1* mRNA in rat brain tissue after hypoxia. Total mRNA (10 μg) from normal rats or rats exposed to hypoxia for 1, 3, or 6 days was loaded in each lane. Blot was hybridized with *flt-1* cDNA probe. 28S ribosome in ethidium bromide-stained gel was used as reference for normalizing total RNA loading.